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Truncated forms of human and simian immunodeficiency virus in infected individuals and rhesus macaques are unique or rare quasispecies

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Abstract

Truncated proviruses of variable sizes are present in peripheral blood mononuclear cells (PBMC) of human immunodeficiency virus type 1 (HIV-1)-infected persons and simian immunodeficiency virus (SIV)-infected rhesus macaques. Here, we investigated whether the highly deleted HIV and SIV proviruses are present in infected organisms as multiple copies or whether each truncated provirus is unique. Using end-point dilution, multiple long-distance (LD) DNA PCR assays were run in parallel using DNA extracted from PBMC of seropositive, treatment-naive persons and from lymph nodes of a rhesus monkey inoculated with cloned, full-length SIVmac239 DNA. The PCR products were titrated and mapped. Most truncated proviruses were present in the DNA samples tested as single, nonintegrated molecules that differed from one another in size and/or nucleotide sequence. These results indicate that truncated primate lentiviral sequences found in infected tissues are unique or rare quasispecies that do not replicate significantly.

Keywords: Defective SIV and HIV-1; Truncated HIV-1 DNA; Truncated SIV DNA; HIV-1 replication in vivo; Lymphoid tissue

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Introduction

Extensively deleted human immunodeficiency virus type 1 (HIV-1) genomes of variable size are present in peripheral blood mononuclear cells (PBMC) of HIV-1-infected persons (Sanchez et al., 1997) and of rhesus macaques inoculated with supercoiled plasmid DNA encoding full-length simian immunodeficiency virus (SIV)mac239 (Pion et al., 2001). In macaques inoculated with a single molecular species of proviral SIV DNA, genomic diversity developed within 4 weeks after inoculation, thus confirming the genetic instability of retroviral genomes in vivo.

In this study, we investigated whether highly deleted HIV-1 and SIV proviruses are present in infected organisms as multiple copies or whether each truncated provirus is present as a single copy. The presence of multiple copies of a given truncated provirus would indicate that it replicates, that the host cell harboring this provirus has divided repeatedly, or that the truncated provirus is formed from full-length viral genomes by recombination that occurs multiple times. Replication of defective HIV-1 and SIV proviruses in integrated forms was demonstrated to coincide with the replication of genomic DNA in dividing cultured cells (Bernier and Tremblay, 1995; Folks et al., 1986; Kim et al., 2001; Little et al., 1994; Trono et al., 1989). In addition, the transcribed RNA of such truncated proviruses may be encapsidated and rescued by helper virus (Berkowitz et al., 1995; Dropulic et al., 1996; Federico et al., 1995; Kaye et al., 1995; Richardson et al., 1993). Resultant virus particles could transduce truncated genomes to other cells. None of these mechanisms has been demonstrated to occur in infected organisms.

Another mechanism to generate multiple copies of a given truncated provirus could be a specific "hot spot" recombination process. Mapping of defective genomes in HIV-1-infected individuals showed that the frequency of deletions is proportional to their proximity to the central part of viral genome, which is consistent with a deletion mechanism involving a single polymerase jump during reverse transcription (Sanchez et al., 1997). This mechanism showed no sequence specificity, as judged from the variable patterns of highly deleted proviral bands detected in different infected individuals (Sanchez et al., 1997).

The variability of extensively deleted proviruses was also studied in PBMC of HIV-1-infected, antiviral drug-naive individuals, who were long-term nonprogressors (LTNP) from the French prospective asymptomatic long-term (ALT) cohort (Candotti et al., 1999b), and in the lymph nodes of rhesus macaques inoculated with full-length SIVmac239 DNA (Liska et al., 1999; Pion et al., 2001). Viral load, which was the only significant variable parameter in the ALT cohort, directly correlated with the quantity of replication-competent virus in PBMC (Candotti et al., 1999a, 1999b). In the same cohort, the viral load was inversely proportional to the levels of HIV-1-specific IgG2 antibodies and helper T cell type 1 (Th1) response (Ngo-Giang-Huong et al., 2001). Because the formation of defective viruses is generally the consequence of frequent serial passages at high multiplicities of infection (Barrett and Dimmock, 1986; Huang and Marmur, 1970; Roux et al., 1991; Sekellick and Marcus, 1980), we assumed that multiple copies of highly deleted proviruses could be found more frequently in PBMC in individuals with high viral loads than in individuals with low viral loads. For the same reason, multiple copies of highly deleted fragments might be more abundant in lymphoid tissue, where both HIV-1 and SIV preferentially replicate and where more replication-competent helper virus is available compared to PBMC (Embretson et al., 1993; Pantaleo et al., 1993; Pion et al., 2001; Smit-McBride et al., 1998; Stahl-Henning et al., 1999; Veazey et al., 1998).

Our experimental approach was based upon an assay to detect identical copies of truncated proviruses in multiple parallel long-distance (LD) DNA PCR runs followed by Southern blot hybridization (Sanchez et al., 1997). We demonstrated that truncated proviruses are not artificially generated during amplification reactions but are present in sample DNA before its amplification; thus, our LD-DNA PCR technique was reliable. Because retrovirus replication critically depends on provirus integration into the host genome, we tested integration of truncated proviruses by a modified Alu sequence-based PCR strategy. Our results suggest that most truncated lentiviral DNA fragments are not integrated and represent unique or rare sequences.

Results

Truncated proviruses contain virus-specific sequences

First, we sought to establish that DNA bands smaller than full-length provirus represent truncated proviral sequences rather than nonspecific PCR artifacts. To this end, LD-DNA PCR products amplified from PBMC DNA of an HIV-1-infected individual were cloned into plasmid pNL4-3 (Sanchez et al., 1997), and the resulting $p\Delta_{CESMO}$ plasmid was sequenced from the 5′ noncoding sequence (NCS) to the *nef* gene, across the deleted regions (Fig. 1). Compared with the parental NL4-3 sequence, $p\Delta_{CESMO}$ contained a simple deletion of 7695 nucleotides flanked at either junction by direct repeats of the four nucleotides AGCT. Short identical stretches flanking the deletion point are characteristic of simple deletions within retroviral genomes (Omer et al., 1983; Pathak and Temin, 1990). The remainder of the nucleotide sequences did not differ significantly from the HIV-1 NL4-3 genome. Similar results were obtained from LD-DNA PCR products amplified from two other HIV-positive individuals (not shown) (Sanchez et al., 1997). Thus, we clearly established that short LD-DNA PCR products represent specific viral sequences present in infected cells.

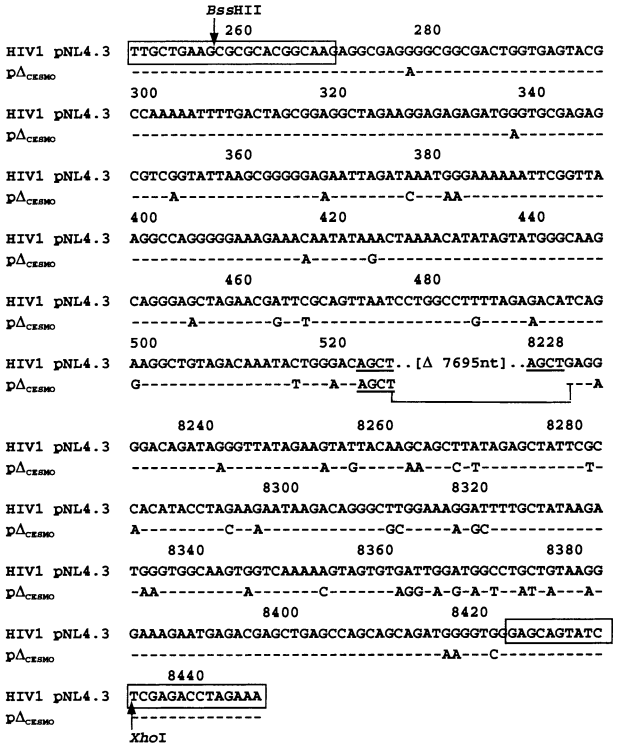


Fig. 1. DNA sequence of cloned truncated provirus $p\Delta_{CESMO}$. The DNA sequence determined from plasmids $p\Delta_{CESMO}$ using primers LTR(U5) and LTR(R) was compared with the corresponding sequences of HIV-1 NL4-3. Numbers in parentheses correspond to the length of sequence deleted from HIV-1 NL4-3. The directly repeated sequences from HIV-1 NL4-3 and truncated proviruses are underlined. Nested primers 5′NCS and *nef* are framed and cloning sites *Bss*HII and *Xho*I are shown by arrows. For localization of primers, see Fig. 2. The length of LD-DNA PCR product amplified by primary primers LTR(U5) and LTR(R) corresponded to 1333 nt. The homology between the sequenced region and HIV-1 NL4-3 sequence upstream and downstream from the deletion junctions corresponded to 95.4 and 87.2%, respectively.

Extensively deleted HIV-1 sequences in PBMC DNA of infected individuals are unique or rare quasispecies

Next, we tested whether extensively truncated fragments of HIV proviruses are present in infected PBMC as multiple copies or as unique quasispecies. Toward this end, six aliquots of DNA extracted from PBMC of a single patient were amplified in parallel at end-point dilution, using primer pairs LTR(U5)/LTR(R) for three aliquots, or 5′NCS/*nef* for the other three, and were then analyzed by Southern blot hybridization (Fig. 2). Among 15 bands obtained by using primers LTR(U5)/LTR(R) (Figs. 2A and B, reactions 1, 2, 3), only two 5.7-kb bands (reactions 1 and 2) had the same hybridization profile. Two additional 0.6-kb fragments (Fig. 2B, reactions 2, 3, line 0.6 kb) were detected only with the LTR(U3) probe, suggesting that they corresponded to 2-LTR circles. Repeated LD-DNA PCRs with primers 5′NCS/*nef* resulted in the amplification of unique fragments in most parallel runs (Figs. 2A and B, reactions 4, 5, 6). Only two 1.4-kb bands (reactions 4 and 5) were the same. Thus, the hybridization profiles of a total of 29 distinct subgenomic DNA bands obtained in six parallel assays were mostly different, suggesting that truncated HIV-1 DNAs in PBMC are generally present as unique or rare copies. Despite differences in the fine molecular structure, five truncated HIV-1 DNA fragments were obtained reproducibly in reactions 1, 2, 3, 4, and 6, and six truncated fragments were obtained reproducibly in reaction 5 (Fig. 2B).

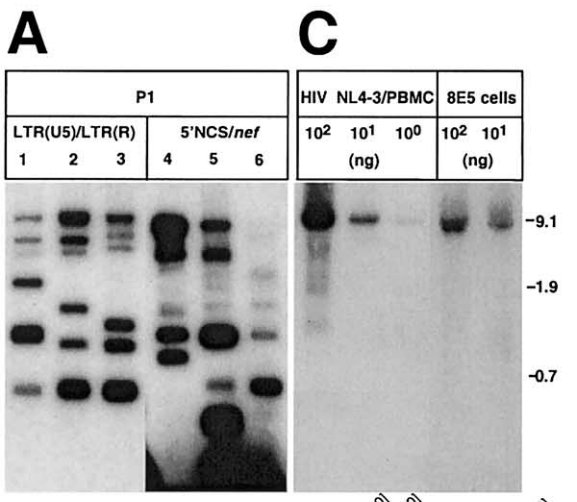
Physical mapping of truncated HIV-1 proviral DNA was repeated with similar results for three other HIV-1 seropositive individuals: two AIDS patients and one LTNP (Fig. 3, Table 1). Despite different LD-DNA PCR patterns in each of several parallel amplifications, the number of truncated proviral DNA bands was reproducible within each individual (Table 1).

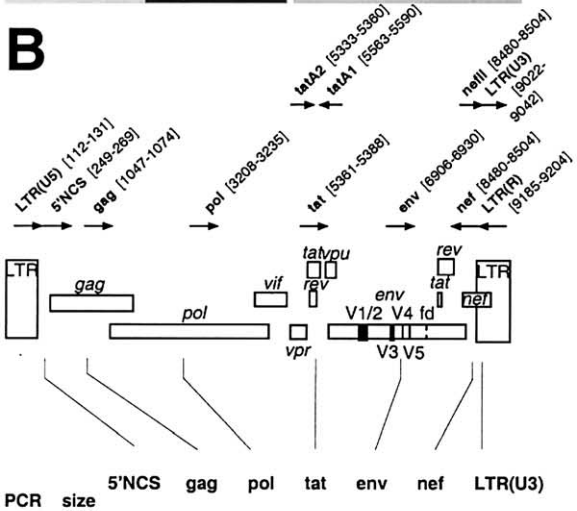
As a control, we examined the pattern of HIV-1 sequences amplified from the PBMC DNA of a healthy donor after cells had been infected in vitro with HIV-1 NL4-3. We also amplified HIV sequences from genomic DNA of cultured 8E5 cells chronically infected with HIV-1 LAV; these cells do not produce infectious virus (Folks et al., 1986). A 9-kb band corresponding to full-length provirus was the major LD-DNA PCR product (Fig. 2C). This result indicates that under the reaction conditions used, the polymerase system faithfully amplified its template, which consisted predominantly of full-size HIV-1 DNA for these in vitro infected cells. Given that full-length LD-DNA PCR products are more difficult to amplify than shorter proviral fragments, these additional control experiments validate our detection of multiply deleted proviral sequences in PBMC DNA of infected individuals.

Lymph nodes of a macaque inoculated with SIV plasmid DNA contain truncated proviruses that are unique or rare

Next, we sought to confirm our findings in a well-defined primate model; we asked whether truncated proviruses could be detected in lymph nodes of a macaque that had become highly viremic after inoculation with cloned SIVmac239 plasmid DNA. We tested whether identical truncated SIVmac239 DNA molecules could be detected reproducibly by mapping of LD-DNA PCR products, or whether truncated proviruses are present as unique species. At limiting dilutions, six aliquots of DNA extracted from lymph nodes of macaque 270W (Liska et al., 1999; Pion et al., 2001) were amplified in parallel using primer pairs SIV-C/SIV-*nef* and analyzed by Southern blot hybridization (Fig. 4).

We observed a similar pattern when comparing ethidium bromide staining (Fig. 4A) with Southern blot hybridization (Fig. 4B) of the same gel visualized by a random prime-labeled probe containing the complete SIVmac239 DNA sequence, indicating that most LD-DNA PCR products are virus specific. Most bands visualized by a random prime-labeled probe were detected also after rehybridization of the same blot with oligonucletide probe SIV-LTR E (Fig. 4C





PCR	size (kb)	5'NCS	gag	pol	tat	env	nef	LTR(U3)
1	9.1	+	+	+	+	+	+	+
	5.7	+	+	+	-	-	-	+
	2.9	+	+	+	-	-	-	+
	1.9	+	+	-	-	-	-	+
	1.1	+	-	-	-	-	-	+
	0.6	+	-	-	-	-	-	+
2	9.1	+	+	+	+	+	+	+
	5.7	+	+	+	-	-	-	+
	3.7	-	-	-	-	+	+	+
	1.4	+	-	-	-	-	+	+
	1.0	+	-	-	-	-	+	+
	0.6	-	-	-	-	-	-	+
3	9.1	+	+	+	+	+	+	+
	6.4	+	-	+	+	+	-	+
	4.8	+	+	+	-	-	-	+
	1.3	-	-	-	-	-	+	+
	0.9	+	-	-	-	-	-	+
	0.6	-	-	-	-	-	-	+
4	8.3	+	+	+	+	+	+	
	4.9	+	+	-	-	-	+	
	1.4	+	-	-	-	+	+	
	1.1	+	-	-	-	-	+	
	0.8	+	-	-	-	-	+	
	0.3	+	-	-	-	-	+	
5	8.3	+	+	+	+	+	+	
	5.0	+	-	-	+	+	+	
	2.0	+	-	-	-	+	+	
	1.4	+	-	-	-	+	+	
	1.1	+	+	-	-	+	+	
	0.7	+	-	-	-	-	+	
	0.4	+	-	-	-	-	+	
6	8.3	+	+	+	+	+	+	
	7.0	+	-	-	+	+	+	
	2.0	+	-	-	+	+	+	
	1.4	+	-	-	-	-	+	
	1.1	+	-	-	-	+	+	
	0.7	+	-	-	-	-	+	

Fig. 2. Mapping of truncated forms of HIV-1 DNA. (A) Electrophoretic mobility of HIV-1 DNA fragments of patient P1 amplified in triplicate by primer pairs LTR(U5)/LTR(R) or 5′NCS/*nef* visualized by Southern blot hybridization with ^{32}P-labeled LTR(U3) or *nef* oligonucleotide probe, respectively. The numbers denote parallel LD-DNA PCRs. (B) Maps of truncated viruses amplified in six parallel LD-DNA PCRs (nos. 1–6). The organization of HIV-1 genome is shown in the top of the panel. Arrows indicate the primers and their orientation. Numbers in parentheses correspond to nucleotide positions in the HIV-1 LAI sequence. Hybridization results for respective probes are shown for each reaction by +, positive signal, and −, no signal. The size of truncated genomes was estimated from their electrophoretic mobility. (C) HIV-1 DNA present in PBMC infected in vitro with HIV-1 NL4-3 and from 8E5 cell line chronically infected with HIV-1 LAV. The resulting product was probed by ^{32}P-labeled *nef* oligonucleotide. The total amount of DNA was adjusted to 1 μg by DNA extracted from PBMC cells from a healthy donor.

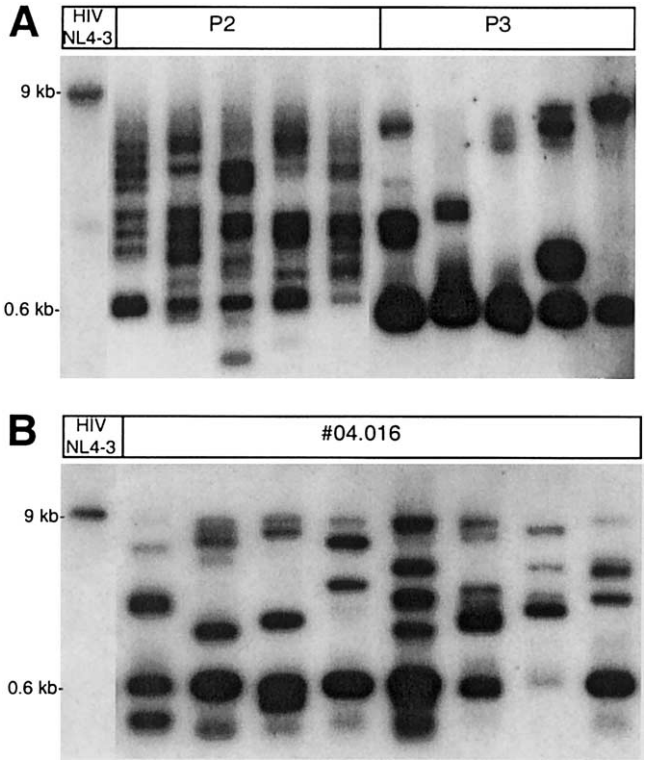


Fig. 3. Diversity of deleted HIV-1 DNAs in PBMC of infected individuals. Repeated LD-DNA PCR of 0.2 μg of DNA of patients P2 and P3 (A) and LTNP no. 04.016 (B) visualized by Southern blot hybridization with ^{32}P-labeled LTR(U3) oligonucleotide probes. Amplification product of the genomic DNA of PBMC infected in vitro with HIV-1 NL4-3 was used as a control.

and E), indicating that LTR sequences are present in most shortened proviruses. The intensity of the radioactive signal obtained with the random prime-labeled probe is proportional to the sizes as well as copy numbers of the amplified fragments in each band. In contrast, the signal intensity generated by oligonucleotide probe hybridization is proportional only to the molarity of the amplified fragments regardless of their sizes. This is shown by comparing hybridization with the complete SIVmac239 probe (Fig. 4B) to the

Table 1
Intraindividual variance of deleted HIV-1 DNA[a]

Individual	Number of truncated proviral bands per 200 ng of DNA	Number of repeated LD-DNA PCR	Repeated/unique bands[b]
P1	4.3 ± 0.6	3	2/15
P2	8.8 ± 0.8	5	0/44
P3	3.2 ± 1.3	5	0/16
No. 04.016	4.6 ± 1.1	8	0/37

[a] Approximately 200 ng of human DNA, equivalent to 2×10^4 PBMC, were amplified for each LD-DNA PCR. Parallel LD-DNA PCRs of four infected individuals (for P1 see Fig. 2, for P2, P3, and LTNP no. 04.016, see Fig. 3) were analyzed by Southern blot hybridization with LTR(U3) probes, and the average number of bands per LD-DNA PCR was counted.

[b] Repeated bands were defined by the same electrophoretic mobility and hybridization profile in Southern blot analysis as shown in detail in Fig. 2.

signal intensity obtained with probe SIV-LTR E (Fig. 4C), which was inversely proportional to the fragment size. At the selected exposition time of radioactive Southern blot, the full-length SIVmac239 proviruses gave only weak signals with the oligonucleotide probe SIV-LTR E but hybridized strongly with the complete SIVmac239 probe (Fig. 4C). This result shows that the molar concentration of full-length LD-DNA PCR products was lower than that of short fragments and is consistent with our finding that the efficiency of LD-DNA PCR is inversely proportional to the logarithm of the fragment size (Sanchez et al., 1997). Full-length SIV proviruses were readily detected by SIV-*tat* probe (Fig. 4D). Preferential hybridization of the SIV-*tat* probe to high-molecular-weight fragments indicates that, as in PBMC of HIV-1-infected patients (Sanchez et al., 1997), the highest frequency of deletions was detected in the center of the SIV genome.

The hybridization profiles of all 24 distinct subgenomic proviral DNA bands obtained in the six parallel assays and listed in Fig. 4F all differed from one another (insufficient resolution of small bands on the gel does not permit interpretation of the patterns for bands migrating at or below 0.3 kb). We conclude that truncated SIV proviruses are generally present as unique or rare copies, thus confirming our findings in HIV-1-infected individuals.

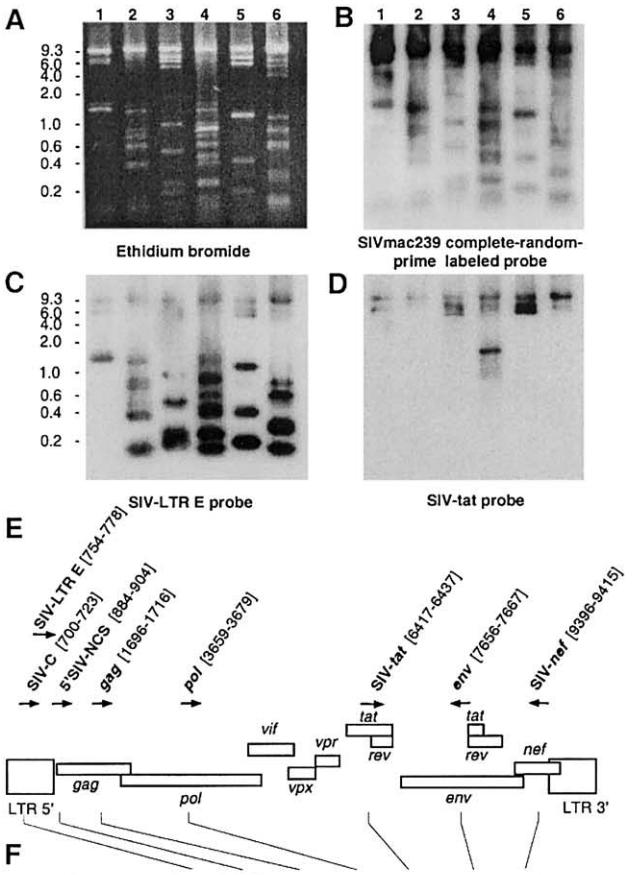
Coamplification with internal standard indicates that truncated HIV-1 proviruses are present in genomic DNA as single molecules

To confirm the uniqueness of truncated molecules, we added an internal standard, pKSK, to each amplification reaction of genomic PBMC DNA of two infected individuals, nos. 04.008 and 06.006. We compared the number of HIV-1 proviruses determined after LD-DNA PCR with the number of pKSK standard molecules amplified in the same reaction (Fig. 5). The initial number of proviruses present in genomic PBMC DNA before amplification was extrapolated from the standard curve generated with the number of pKSK molecules before and after amplification.

The 5.8-kb pKSK standard encoded the target sequences for LD-DNA PCR primers localized within HIV-1 LTR (Fig. 5A, row c). The HIV-1 LTR sequences of the pKSK standard enclosed the non-HIV, pUC18, part of pNL4-3, that was specifically detected by the PUC43 probe (Fig. 5A, row c and d). The number of pKSK copies after PCR was estimated by comparing the intensity of hybridization signal of a single 5.8-kb band with a known quantity of plasmid pNL4-3 directly loaded to the agarose gel (Fig. 5B, lanes g–i). The LD-DNA PCR amplification factor for the pKSK internal standard was then calculated as the ratio of the number of pKSK molecules after LD-DNA PCR reaction to the known quantity of input pKSK molecules introduced into the reaction. In a similar way as for the pKSK standard, the number of HIV-1 proviruses after LD-DNA PCR was estimated by comparing the intensity of the hybridization signal, generated with the HIV-1-specific 5′NCS probe, with the known quantity of the pNL4-3 gel standard (Fig. 5C). Both probes PUC43 and 5′NCS matched the pNL4-3 gel standard (Figs. 5B and C, lanes h and i), thus serving as a hybridization standard compensating for the efficiency of rehybridization with different probes. Reprobing of the Southern blot with the 5′NCS oligonucleotide probe revealed DNA fragments of different sizes in each of the parallel PCR reactions (Fig. 5C). The fact that the internal standard amplified as a single band confirms that the variable profile of PBMC DNA amplification is not a LD-DNA PCR artifact, but that different truncated proviruses are found in each of the amplified aliquots.

The estimated number of truncated proviruses present in genomic PBMC DNA before amplification corresponded to a single molecule (Fig. 5D). In these calculations, we did not consider that the efficiency (ε) of amplification by LD-DNA PCR is dependent on the length of the target sequence ($\varepsilon = 53 - 13 \times \log$ bp; Sanchez et al., 1997). When the LD-DNA PCR efficiency of the longest DNA fragment (Fig. 5C, band 6, size $\approx$ 6 kb, $\varepsilon = 4.2$) and of the pKSK standard (size $\approx$ 5.8 kb, $\varepsilon = 4.1$) was compared to that of the shortest one (Fig. 5C, band 5, size $\approx$ 0.7 kb, $\varepsilon = 16.3$), the variability of ε did not exceed a factor of 4 (Fig. 5D). This factor was neglected considering that the assessed numbers of HIV-1 molecules before amplification varied from 0.3 to 1.5, regardless of their molecular weight (Fig. 5D). However, these factors can reach highly significant values (>16-fold for band 5, size $\approx$ 0.7 kb) when truncated forms are compared with full-length proviruses, amplified with $\varepsilon = 1$ (Sanchez et al., 1997). Indeed, 100 copies of the full-length HIV-1 genome amplified about 10^8 times (Fig. 5C, lane g), reaching the same intensity as 10^{10} molecules of the pNL4-3 gel standard (Fig. 5C, lane h), whereas the truncated forms of HIV-1 proviruses amplified $>10^9$ times.

Taken together, both assays, one based on the mapping of truncated molecules and the other on their coamplifica-



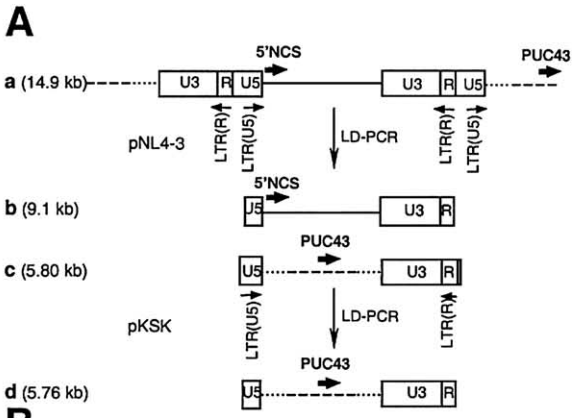
tion with an internal standard, indicate that truncated HIV-1 DNA in PBMC of infected individuals are unique or rare quasispecies. Similar results were obtained in AIDS patients (Figs. 2 and 3) and LTNPs (Fig. 3).

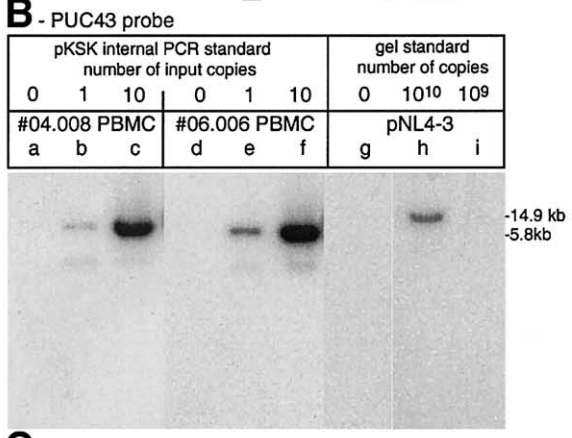
Deletions in proviral DNA are most prevalent in the center of the HIV-1 genome and are present before LD-DNA PCR of PBMC DNA from infected individuals

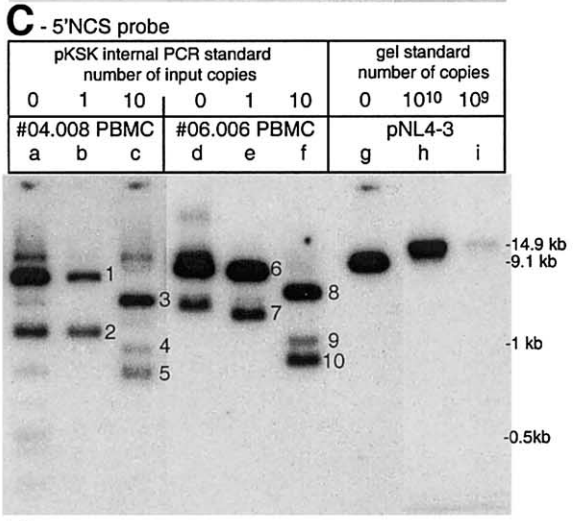
In previous control experiments, we demonstrated that the major LD-DNA PCR products in primary lymphocytes infected in vitro consisted of full-length HIV-1 or SIV sequences (Fig. 2C) (Pion et al., 2001; Sanchez et al., 1997). This finding was in contrast to our results obtained by amplification of PBMC DNA from infected persons or monkeys. However, it is still possible that HIV-1 DNA from PBMC of infected individuals is more susceptible to recombination by LD-DNA PCR than HIV-1 DNA from in vitro cultivated cells. To verify the presence of deleted molecules in PBMC, we tested whether one of the general consequences of the deletion mechanism, a high frequency of deletions in the center of HIV-1 genome (Sanchez et al., 1997), was detectable before LD-DNA PCR. Standard semiquantitative PCR with primer pairs *tat*A2/*tat*A1 and *nefII*/LTR(R) (Fig. 2B) was used to compare the titers of HIV-1 sequences from the central and terminal parts of the HIV-1 genome, respectively (Fig. 6A). In PBMC DNA of 13 LTNPs with plasma viral RNA loads ranging from <800 to 480,000 copies per milliliter, the mean titer of the terminal *nef*-LTR sequences was 5.5 ± 5.0-fold higher ($P = 0.0017$) than that of the central *tat* sequences.

In a second assay, we tested whether cleavage of HIV-1 DNA in its central region would prevent amplification of full-length and long subgenomic fragments yet have no effect on the amplification of shorter products. Restriction sites of endonucleases that rarely cleave the HIV-1 provirus were mapped in genomic DNA from 10 individuals with the aim of finding an enzyme that only cleaves the center of the viral genome. The result of LD-DNA PCR performed before and after restriction cleavage of PBMC DNA of LTNP no. 04.016 by *Eco*RI is shown in Fig. 6B. HIV-1 DNA of

Fig. 4. Physical mapping of extensively deleted forms of SIV DNA extracted from the lymph nodes of macaque 270W inoculated with supercoiled plasmid DNA encoding the intact SIVmac239 genome. (A) Electrophoretic mobility of SIV DNA fragments at limiting dilution amplified in six parallel reactions by primer pairs SIV-C/SIV-*nef* visualized by ethidium bromide. (B–D) Southern blot hybridization with either ^{32}P-random prime-labeled probe generated from the complete SIVmac239 genome (B), SIV-LTR E oligonucleotide probe (C), or SIV-*tat* oligonucleotide probe (D). (E) The organization of the SIV genome. Arrows indicate the primers and their orientation. Numbers in parentheses correspond to nucleotide positions in the SIVmac239 sequence. (F) Physical maps of truncated proviruses amplified in six parallel LD-DNA PCRs (nos. 1–6). Hybridization results for respective probes are shown for each reaction by +, positive signal, and −, no signal. The size of truncated genomes was estimated from their electrophoretic mobility.







D

band	size (kb)	LD-DNA PCR efficiency (ε)	number of HIV-1 proviruses after PCR x 10^9	pKSK amplification factor x 10^9	number of HIV-1 proviruses before PCR
1	5.0	5.2	2.1	3.4	0.6
2	1.5	12.0	1.8	3.4	0.5
3	2.5	9.1	3.2	2.9	1.1
4	1.0	14.3	0.9	2.9	0.3
5	0.7	16.3	1.2	2.9	0.4
6	6.0	4.2	11.0	7.6	1.5
7	2.0	10.4	2.8	7.6	0.4
8	3.5	7.2	5.6	4.3	1.3
9	1.1	13.6	1.4	4.3	0.3
10	0.8	15.6	2.3	4.3	0.5

Fig. 5. Coamplification by LD-DNA PCR of HIV-1 DNA with the internal standard. (A) Schematic representation of pNL4-3 (a, b) and an internal standard pKSK (c, d) linearized by *Aat*II and *Hin*dIII, respectively. LD-DNA PCR products amplified by primers LTR(U5)/LTR(R) (b, d). Solid, dotted, and dashed lines show HIV-1, human flank, and pUC18 sequences, respectively (not in scale). Bold arrows represent complementary sequences for PUC43 or 5′NCS oligonucleotide probes, specific for internal standard and HIV-1 DNA, respectively. (B, C) Aliquots of 200 ng of DNA extracted from PBMC of LTNPs nos. 04.008 and 06.006 were coamplified with none (a, d), 1 (b, e), and 10 (c, f) copies of pKSK using LD-DNA PCR primers LTR(U5) and LTR(R). The same membrane was hybridized with ^{32}P-labeled PUC43 (B) and 5′NCS (C) oligonucleotide probes. One hundred input molecules of pNL4-3 were amplified in control LD-DNA PCR without internal marker (g). A total amount of 10^{10} (h) and 10^9 (i) molecules of pNL4-3 linearized by *Aat*II was loaded onto the agarose gel without previous PCR amplification (gel standard). It was used as external hybridization standard to determine the number of pKSK (B) and HIV-1 DNA (C) molecules after LD-DNA PCR. Numbers in (C) show the bands in which the amount of input molecules was determined. (D) Titration of truncated HIV-1 proviruses in the PBMC of infected individuals. The size-dependent amplification efficiency of LD-DNA PCR (ε) was calculated according to the previously published formula $\varepsilon = 53 - 13 \times \log$ bp (Sanchez et al., 1997). The relative amplification efficiency of full-length HIV-1 provirus according to this formula equals one. The amplification factor is the ratio of pKSK molecules after and before LD-DNA PCR. The number of proviral HIV-1 DNA molecules introduced into the LD-DNA PCR was calculated as the ratio of the number of HIV-1 proviruses after LD-DNA PCR to the pKSK amplification factor.

this individual was cut at positions 2.5 and 5.3 kb in the standard HIV-1 map. The amplification of the full-length and long subgenomic HIV-1 DNA fragments, detected by hybridization with *tat* probe, was prevented by prior *Eco*RI cleavage. Bands became detectable when the same membrane was rehybridized with the LTR(U3) probe. Thus, both assays suggest that deleted HIV-1 proviruses are present in PBMC DNA before its amplification by LD-DNA PCR and are not artifacts of this PCR strategy.

Truncated proviruses are not integrated in PBMC DNA of HIV-1-infected individuals

To test whether truncated proviruses were integrated into the host genome, we used a modified Alu sequence-based PCR strategy (Fig. 7). In this modification, the junction between the nearest Alu sequence and the HIV-1 provirus was amplified by the primer pair Alu/*nef*. This was followed by second-round PCRs with primers designed to amplify either the LTR or the *tat* regions (Fig. 7A); the final PCR products were visualized by hybridization for either the terminal or the central proviral segments. We assumed that due to the higher efficiency of PCR amplification of shorter fragments, integrated forms of truncated proviruses would be amplified preferentially compared to integrated forms of full-length proviruses (Pion et al., 2001; Sanchez et al., 1997, 1998). Therefore, if truncated proviruses were integrated, the higher titers of HIV-1 sequences from the terminal as compared to the central parts of the HIV-1 genome (Fig. 6A) should be even more pronounced following Alu/

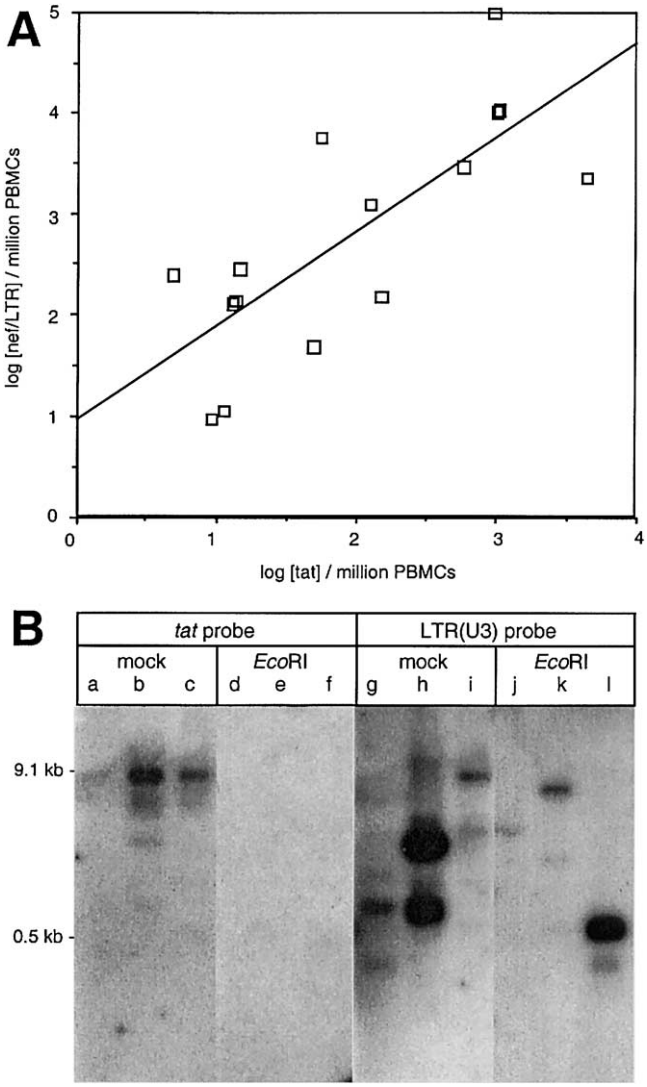


Fig. 6. The highest frequency of deletions in the center of the HIV-1 genome is detected in PBMC before LD-DNA PCR. (A) Titers of HIV-1 sequences from the central and from the terminal parts of HIV-1 genome per million cells were determined by standard semiquantitative PCR of end-point dilution of DNA extracted from PBMC using primer pairs *tat*A1/*tat*A2 and *nef*II/LTR(R). For localization, see Fig. 2B. An equivalence of 1 μg of human DNA to 10^5 PBMC was used to calculate cellular virus load. (B) Sensitivity of LD-DNA PCR to cleavage by restriction endonuclease *Eco*RI. PBMC DNA of LTNP no. 04.016 was cleaved (d–f, j–l) or mock-treated (a–c, g–i) with 15 units of restriction endonuclease *Eco*RI per microgram of DNA at 37°C for 4 h and amplified in triplicate (200 ng of DNA per aliquot) by LD-DNA PCR using primer-pair LTR(U5)/LTR(R). Southern blot was hybridized and rehybridized with ^{32}P-labeled LTR(U3) and *tat* probes, respectively. *Eco*RI sites in the major HIV-1 sequence of subject no. 04.016 were localized around positions 2.5 and 5.3 kb of standard HIV-1 map.

*nef*PCR (Fig. 7A). In contrast, if truncated proviruses are not integrated, Alu/*nef*PCR will only generate first-round product from integrated, full-length proviruses, which will yield equal titers of terminal and central sequences after the second rounds of PCR and hybridization with probes for the LTR or *tat*.

The titers of the terminal and central HIV-1 sequences following Alu/*nef*PCR were determined for two LTNP, no. 04.016 (viral load, 4.8×10^5 copies/ml of plasma) and no. 09.006 (viral load, 4×10^4 copies/ml of plasma). Without amplification by Alu/*nef*PCR, the titer ratios ($R_{t/c}$) of the terminal to the central HIV-1 sequences were $R_{t/c} = 10$ for LTNP no. 04.016 and $R_{t/c} = 5$ for LTNP no. 09.006. Following Alu/*nef*PCR, the same titers of LTR and *tat* sequences were found in the amplification products. This shows that Alu/*nef*PCR preferentially amplified full-length proviruses that contain *tat* and suggests that most truncated HIV-1 proviruses are not integrated.

Discussion

Here we showed that truncated HIV-1 or SIV proviruses detected in infected human or primate hosts are unique or rare quasispecies that do not significantly replicate. The uniqueness of the genomic structures of truncated proviruses amplified in multiple parallel LD-DNA PCR runs was demonstrated. Mapping showed that each truncated provirus was present in PBMC and lymph node DNA only once, as a single molecule. Titration of truncated HIV-1 proviruses with an internal LD-DNA PCR standard confirmed this conclusion.

Our studies showed that LD-DNA PCR faithfully amplified the template DNA and did not generate artificial recombinations. The frequency of recombination by *Taq* polymerase during PCR is less than 5% (Meyerhans et al., 1990) and is probably further reduced with the *Pwo* polymerase in the LD-DNA PCR system. Such a low recombination frequency cannot explain the presence of extensively deleted molecules in PBMC and lymph nodes DNA. The relative intensity of deleted bands was most frequently inversely proportional to their size, in a similar way as shown previously for pairs of competing plasmids of different size (Sanchez et al., 1997, 1998). However, many fragments did not follow this general rule, especially when comparing bands migrating closely together. Most likely, both competition among truncated fragments and variability of target sequences for PCR primers and oligonucleotide probes were responsible for the varying intensity of amplified bands.

We have studied the replication of HIV-1 in infected individuals and of SIV in infected macaques by focusing on the formation of truncated proviruses. Taken together, these results provide further evidence that the majority of truncated molecules are randomly formed during reverse transcription of lentiviral genomes in infected cells. Thus, although replication of defective proviruses was repeatedly demonstrated in in vitro systems, it was not detected in our experiments in which virus replication in vivo was assayed. In the great majority, only single copies of truncated proviruses were found in lymph nodes of SIVmac239-infected macaques, although SIV replicates extensively in this tissue (Smit-McBride et al., 1998; Stahl-Hennig et al., 1999; Vea-

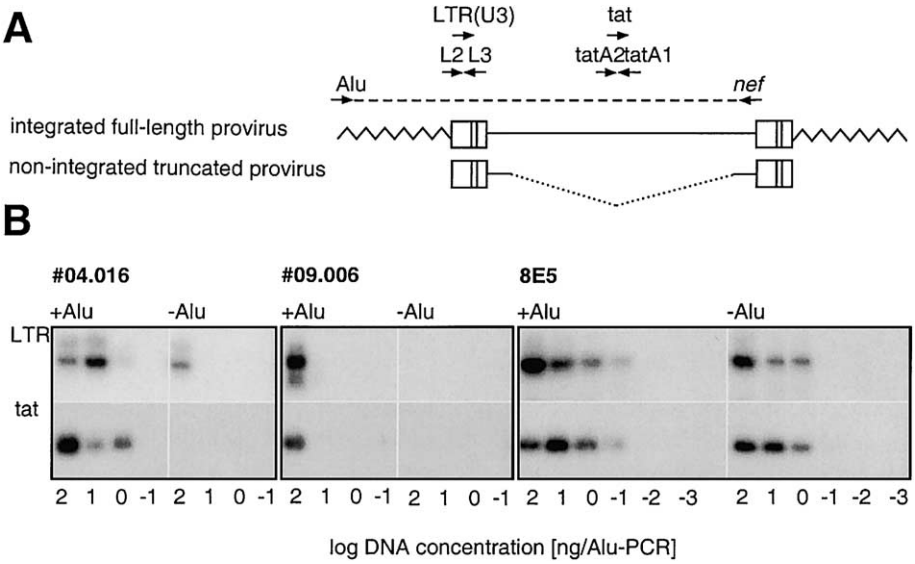


Fig. 7. Unintegrated truncated proviruses in PBMC of LTNP individuals. (A) Schematic representation of modified Alu sequence-based PCR strategy to detect integration of truncated proviruses into the host genome. PCR amplifications (Alu/*nef*) of existing Alu-HIV provirus junctions (dashed line) were subjected to a second round of PCR with HIV-1-specific primers from the terminal (L2/L3) and the central (*tat*A2/*tat*A1) parts of the HIV-1 genome. Full-length provirus is represented as integrated into the human genome (zigzag line). Truncated, nonintegrated provirus is shown by a dotted line. (B) Amplification products of Alu/*nef*PCR of DNA extracted from PBMC of LTNP no. 04.016 and 09.006 contained the same amount of the terminal (LTR) and central (*tat*) sequences. Genomic DNA isolated from PBMC of two LTNP was amplified by Alu/*nef*PCR followed by a second round of PCRs using either the primer pairs L2/L3 or *tat*A2/*tat*A1. Amplification products were visualized by Southern blot hybridization with ^{32}P-labeled oligonucleotide probes LTR(U3) (for L2/L3) and *tat* (for *tat*A2/*tat*A1). 8E5 cells used as positive controls contain a single integrated HIV-1 genome.

zey et al., 1998). Thus, the higher quantity of full-length virus present in lymph nodes in comparison with PBMC (Pion et al., 2001) is not sufficient to provide a helper function for replication of highly deleted forms. Similarly, only single copies of truncated HIV-1 proviruses were detected in the patients with a high viral load.

Integration of viral DNA into the host cell genome and subsequent superinfection with a helper virus are both necessary for the replication of truncated proviruses. By using a modified Alu sequence-based PCR strategy, we have shown that most truncated proviruses in PBMC of HIV-1-infected individuals are not integrated. Free viral DNA is present in PBMC in nonstable form and is rapidly degraded with a half-life of about 1 day (Sharkey et al., 2000; Zack et al., 1990). We have recently shown in a longitudinal follow-up study of SIVmac239-infected macaques that truncated proviruses do not continuously accumulate over the period of infection in vivo (Pion et al., 2001). Instead of continuous growth, the titers of deleted proviruses followed titers of full-length SIVmac239 DNA, including their decline after the peak of primary viremia. This provides further evidence that truncated proviruses do not replicate and indicates a limited life span and dynamic renewal of lymphocytes harboring truncated proviruses.

Our results suggest that truncated HIV genomes are only rarely encapsidated—if at all—and therefore, do not significantly contribute to the formation of defective interfering particles. Thus, possible interference seems to be limited to interactions of truncated forms resident when infected cells are superinfected with replication-competent helper virus. Recent data have shown that HIV *nef* and *tat*, both of which play important roles in lentiviral pathogenesis, can be expressed from unintegrated viral genomes (Wu and Marsh, 2001). Nef coding sequences were present in most truncated proviruses. Besides down-modulation of CD4-receptor and interference with superinfecting virus, *nef* expression might protect cells harboring truncated proviruses against cytoxic T lymphocyte killing by down-regulation of MHC I molecules. Further quantitative studies in carefully selected groups of HIV-1-infected patients presenting different virus loads and different rates of progression towards AIDS will be necessary to evaluate the biological significance and the potential of truncated viruses for viral interference.

Materials and methods

Subjects

Seropositive individuals, who were anti-retroviral therapy-naive, gave their informed consent. These individuals were in the primary phase of HIV-1 infection or belonged to the “ALT” cohort of LTNPs (Candotti et al., 1999a,b). Criteria for inclusion in the ALT group were HIV-1 infection for no less than 8 years, an asymptomatic clinical status, and a nondecreasing CD4 cell count $\geq$600/mm^3 during the last 5 years. Plasma viral loads were determined at the time of blood sampling.

Animal care and DNA inoculation of monkey

The experiments with adult rhesus monkey 270W were approved by the Animal Care and Use Committees at Bioqual, Inc. (Rockville, MD) and the Dana-Farber Cancer Institute (Boston, MA). Ketamine anesthesia was used before all procedures that required the removal of the animal from its cage. Macaque 270W was inoculated intramuscularly with 500 μg of supercoiled pBSIVmac239 as described (Liska and Ruprecht, 1999).

DNA extraction

PBMC ($\geq 10^6$ cells) or lymph nodes biopsies (about 50 μg) were frozen and kept at -70°C until DNA extraction. High molecular weight genomic DNA was isolated as described (Liska and Ruprecht, 1999; Sanchez et al., 1997). PCR amplification of the β-actin gene was used to test the ability of the DNAs to be amplified and to ensure that approximately equal amounts of genomic DNA were included in the reactions.

PCR amplification of viral DNA

To amplify HIV-1 or SIV sequences, DNA samples in a background of 100 ng of carrier human PBL DNA were subjected to LD-DNA PCR in a 50 μl reaction mixture containing 50 mM Tris–HCl, pH 9.2, 14 mM $(NH_4)_2SO_4$, 1.75 mM $MgCI_2$, 350 μM each dNTP and 45 pmol each of LTR(U5) or 5′NCS or SIV-C (sense) and LTR(R) or *nef* or SIV-*nef* (antisense) primers (Figs. 2B and 4E), with 1.75 units of enzyme (Expand Long Template PCR system, Roche, Basel, Switzerland) as described (Pion et al., 2001; Sanchez et al., 1997). Viral origin (HIV-1 or SIV) is indicated in the nomenclature of all SIV but no HIV-1 primers. Reaction mixtures were then subjected to 40 amplification cycles. The first 10 amplification cycles comprised a denaturing step of 10 s at 94°C, a primer-annealing step of 30 s at 62°C, and a primer extension step of 8 min at 68°C. The primer extension step of the following 15 amplification cycles was gradually extended by 20 s at each cycle and kept constant at 13 min for the last 15 cycles. A final extension step of 7 min at 72°C was performed. In addition to the whole internal sequence of HIV-1 provirus, LTR(U5) and LTR(R) primers span the LTR-LTR junction of the two-LTR circle and may generate a shorter amplification product (595 nucleotides in the case of HIV-1 LAI).

The quantity of DNA molecules bearing either central or terminal sequences of the HIV-1 genome was estimated by semiquantitative PCR of *tat* and *nef*/LTR genes, respectively. Tenfold dilutions of DNA were assayed in a standard PCR reaction with *Taq* polymerase and *tat* A1 primer, 5′-TGAGGAGGTCTTCGTCGCTGTCTCCGCT-3′ (nt 5563–5590, antisense) and *tat* A2 primer, 5′-TTGGGTGTCGACATAGCAGAATAGGCGT-3′, (nt 5333–5360, sense) for the presence of a 232-nt amplification product or *nef*II primer, 5′-GGAGCAGTATCTCGAGACCTGGAAA-3′ (nt 8480–8504, sense) and the antisense LTR(R) primer for the presence of 726 amplification product (Sanchez et al., 1997; Sauvaigo et al., 1993; Fig. 2B). Serial 10-fold dilutions of plasmid pNL4-3 standard DNA were amplified in the control experiment.

Southern blotting

PCR products were transferred to a Hybond N^+ membrane (Amersham, Life Science) and hybridized with ^{32}P-labeled oligonucleotide probes 5′NCS, *gag*, *pol*, *env*, *nef*, LTR(U3), *tat* 5′-TTCCTGCCATAGGAGATGCCTAAGGCTT-3′ (nt 5561–5588, antisense) and SIV-E as described previously (Pion et al., 2001; Sanchez et al., 1997) (Figs. 2B and 4E). In reprobing experiments, ^{32}P-labeled oligonucleotides were stripped off from Hybond-N^+ membrane in 0.1× SSC, 1% SDS at 70°C for 30 min. Hybridization was repeated up to eight times without a detectable loss of sensitivity. [^{32}P]-dCTP-labeled probe containing complete SIVmac239 sequences ($>1 \times 10^9$ dpm/μg) was prepared by Megaprime DNA Labelling System (Amersham Biosciences, France) using as a template the viral part of the plasmid pSIVmac239 amplified by LD-DNA PCR and primer pairs SIV-C/SIV-*nef*.

Cloning of HIV-1 DNA fragments

LD-PCR products were separated by agarose gel electrophoresis, electroeluted, and reamplified by LD-PCR using the nested primers 5′NCS and *nef* to obtain sufficient quantities of DNA for molecular cloning. The primers 5′NCS and *nef* contain *Bss*HII and *Xho*I restriction sites, respectively, conserved in HIV-1 nucleic acid sequences database. LD-PCR products were gel-purified, digested with *Bss*HII and *Xho*I to generate cohesive ends, and inserted by homologous recombination into plasmid pNL4-3 (Sanchez et al., 1997).

Construction of the internal standard for LD-DNA PCR

A pKSK plasmid of 5.8 kb was constructed by cleavage of pNL4-3 by *Sac*I, electropurification, and religation of the vector, resulting in the elimination of the HIV-1 coding sequence. pKSK contained a single HIV-1 LTR surrounded by a non-HIV-1 DNA sequence. Taking into account that shorter templates are amplified by LD-DNA PCR with a higher efficiency than longer ones, the 5.8-kb sequence of the internal standard revealed amplification kinetics similar to those of plasmids bearing deleted ($p\Delta_{CESMO}$, $p\Delta_{FOCEP}$, $p\Delta_{NIPDJ}$) HIV-1 sequences (Sanchez et al., 1997, 1998).

Titration of HIV-1 DNA deleted sequences by coamplification with an internal standard

The DNA extracted from PBMC of infected persons was coamplified by LD-DNA PCR with an internal standard pKSK, previously linearized by digestion with *Hin*dIII in the LTR. Linearization precluded a possible bias in the amplification of the mixture of circular and linear forms present in plasmid preparations. pKSK were specifically detected by the ^{32}P-labeled oligonucleotide PUC43 probe, 5′-CGGTGCTACAGAGTTCTTGA-3′, corresponding to the pUC sequence (1257–1276, sense, Fig. 5A, rows c and d), but lack sequences homologous to HIV-1-specific oligonucleotide probes outside of LTR. The amplification products were separated in an agarose gel together with a known amount of non-PCR-amplified plasmid pNL4-3 containing both HIV-1 and the pUC vector sequences (Fig. 5A, row a). Southern blots were hybridized with the ^{32}P-labeled oligonucleotide 5′NCS HIV-1-specific probe, stripped off, and subsequently rehybridized with the PUC43 probe specifically detecting pUC sequence. The radioactivity in all bands was determined by PhosphoImager (Fuji Inc., Japan). Only limiting concentrations of internal standard (≤10 input pKSK molecules per LD-DNA PCR reaction) were used in this assay to prevent competitive inhibition of HIV-1 DNA amplification by the standard.

Restriction mapping and cleavage of patient HIV-1 DNA

Full-length HIV-1 DNA from PBMC of infected individuals was prepared by LD-DNA PCR using primer pair LTR(U5)/LTR(R) followed by nested primers 5′NCS/*nef*. In the absence of sequence data for the HIV-1 genomes of infected individuals, restriction mapping was performed by single and double cleavage of HIV-1 DNA by the restriction endonucleases *Bcl*I, *Eco*RI, *Nde*I, *Sal*I, and *Stu*I. After determination of restriction sites, PBMC DNA of infected individuals was cleaved with a restriction endonuclease cutting the HIV-1 genome in its central part and not at its extremities.

HIV-1 DNA-integration assay

The Alu-HIV-1 DNA junctions present in PBMC of infected individuals were amplified by primers Alu (sense) (Wu and Marsh, 2001) and *nef* (antisense) (Sanchez et al., 1997); the amplification products were subjected to a second round of PCR with HIV-1 LTR-specific primers L2/L3 (Wu and Marsh, 2001) and *tat* gene-specific primers *tat*A2/*tat*A1. PCR products were visualized by hybridization with ^{32}P-labeled oligonucleotide probes LTR(U3) or *tat* for primer pairs L2/L3 or *tat*A2/*tat*A1, respectively. 8E5 cells used as a positive control were obtained through the AIDS Research and Reference Reagent Program from Dr. T. Folks (Folks et al., 1986).

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